

Changes of three organic acids in the process of Cd and Pb phytoextraction by *Helianthus annuus* L.

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ABSTRACT

In the present study, sunflower was used to enrich the cadmium (Cd) and lead (Pb) in sand culture, and the changes of metals uptake, organic acids, pH, and redox potential were investigated. Results showed that the Cd and Pb accumulation was dissimilar in treatments. In single treatments of Cd or Pb, the amount of glutaric and lactic acids decreased with concentrations; the secretion of glutaric and glycolic acids in 5 mg/L Cd treatments were the highest at the 90th day. In the mixed treatments of Cd and Pb, glutaric acids in cultures with Cd 5 mg/L + Pb 50 mg/L and Cd 10 mg/L + Pb 100 mg/L were higher than those in other treatments. Both lactic and glycolic acids reached the highest values in Cd 10 mg/L + Pb 100 mg/L treatments at the end of experiment. Besides, the Cd or Pb uptake showed various correlations with pH, redox potential, and organic acids, the reason might be that the presence of Cd or Pb influenced the organic acids exudation, and alterations of rhizosphere, including acidity, redox potential, and organic acids, impacted the bioavailability and phytoextraction of toxic metals conversely.

Keywords: sunflower; bioaccumulation; root exudates; heavy metals

The recovery of sites contaminated with heavy metals is one of the major challenges for environmental institutions. Conventional cleanup technologies are generally too costly, and often harmful to desirable soil properties for the restoration of contaminated sites (Ruttens et al. 2010). Recently, bioremediation has been applied widely as a low cost and ecologically-responsible alternative to the expensive physical-chemical methods. Many reports indicated that sunflower could be used as a good candidate for phytoextraction of heavy metals due to its strong tolerance and accumulation ability. Evidences show that sunflower could uptake Cd²⁺ in association with *Pseudomonas putida*, and it was suited to phytoremediation of moderately Pb-contaminated soil when enhanced by nutrients (Wu et al. 2006, Lin et al. 2009).

It was crucial to understand the response of organic acids from root exudates to heavy metals exposure for the successful implementation of phytoremediation technologies (Kim et al. 2010a). Heavy metals could interfere with the physiochemical and biochemical processes in plants, which varied the diversity and amount of organic acids secreted by roots (Rascio and Navari-Izzo 2011). López-Millán et al. (2009) studied the effect of cadmium on the growth of *Lycopersicon esculentum*; results showed that high Cd supply (100 µmol) impeded the citric acid synthesis in roots; however, some metals (Al³⁺, Pb²⁺, and Zn²⁺) were reported to stimulate secretion of malic and citric acids in the rhizosphere of sunflower (Saber et al. 1999). Inevitably, the exudation of metal-inducible organic acid anions would change the toxicity and bioavail-

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ability of heavy metals (Kochian et al. 2005). In the study of Zhang et al. (1997), citric and malic acids in wheat rhizosphere mobilized cadmium in soil and increased Cd contents in shoots; Kim et al. (2010b) suggested that organic acids addition could alleviate the inhibitory effect of Cd and Cu on the growth of *Echinochloa crus-galli*, and made the uptake of metals 2–4 folds higher. Until now, there were relatively few studies on the response of organic acids from sunflower root exudates to cadmium and lead.

Therefore, the objective of our study is to investigate changes of organic acids (glutaric acid, glycolic acid, and lactic acid) from sunflower root exudates when exposed to cadmium and lead. Besides, relationships among Cd and Pb uptake, redox potential, pH, and organic acids were discussed in order to supply some available information for phytoremediation of heavy metals.

MATERIAL AND METHODS

Seed preparation. Seeds of sunflower (*Helianthus annuus* L.) obtained from Shenyang Agricultural University were surface sterilized by immersion in 20% v/v commercial bleach and shaken at 144 r/min on an orbital shaker (Beijing, China) in sterile distilled water for 6 h. Then they were sown onto stainless plate with aseptic gauze in incubator, seeds on plates showing microbial growth were discarded. When the sterile seedlings reached approximately 10 cm, 6 seedlings were transferred to each sterilized apparatus with 4.5 kg heat-sterilized sand. And the experiment was preformed for 90 days.

Experimental design. $\text{CdCl}_2 \cdot 2.5 \text{H}_2\text{O}$ and $\text{Pb}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ (reagent grade) were separately diluted in deionized water, then solutions were added into sand culture and homogenized respectively. The concentrations were designed according to the contaminated soil in the Zhangshi irrigation area, which was polluted by heavy metals for over 30 years. The treatments were as follows: (i) control; (ii) Cd 5, 10, 40 mg/L (Cd5, Cd10, Cd40); (iii) Pb 50, 100, 400 mg/L (Pb50, Pb100, Pb400) and (iv) Cd 5 mg/L + Pb 50 mg/L, Cd 5 mg/L + Pb 100 mg/L, Cd 5 mg/L + Pb 400 mg/L, Cd 10 mg/L + Pb 50 mg/L, Cd 10 mg/L + Pb 100 mg/L, Cd 10 mg/L + Pb 400 mg/L, Cd 40 mg/L + Pb 50 mg/L, Cd 40 mg/L + Pb 100 mg/L, Cd 40 mg/L + Pb 400 mg/L (Cd5 + Pb50, Cd5 + Pb100, Cd5 + Pb400, Cd10 + Pb50, Cd10 + Pb100, Cd10 + Pb400, Cd40 + Pb50, Cd40 + Pb100, Cd40 + Pb400). Controls and treatments were in triplicates for analysis.

Root exudates collection. Root exudates were obtained by aseptic collection apparatus (Yoshitomi and Shann 2001). The apparatuses were arranged randomly in a greenhouse under 14 h light cycles ($110 \mu\text{mol}/\text{m}^2/\text{s}$) and in a temperature range of 18–28°C. A sterile 1/4 strength modified Hoagland and Arnon (1950) nutrient solution was used to irrigate the root zone in the first week, and then nutrient solution was changed to full Hoagland's solution. The humidity of sand substrate was kept at about 30%. Systems were not used if sterility was compromised. The root exudates were collected every 30 days, and immediately filtered, lyophilized (FreeZone 12, Labconco, Kansas City, USA) to 10 mL and stored at –20°C for further analysis.

Organic acids, acidity, and redox potential analysis. Organic acids from root exudates were separated using an ion exchange Supelcogel C-610H, (Supelco, Gland, Switzerland) column (30 cm by 7.8 mm). The mobile phase was 10.0 mm H_3PO_4 at a flow rate of 0.8 mL/min. Separation was carried out at 30°C. The wavelength of UV detector was 210 nm (GB/T 5009.157-2003). The acidity and redox potential in sand culture were measured by pH meter (Sigma, Wheaton, USA) and ORP meter (Jenco, California, USA), respectively.

Determination of biomass, cadmium and lead. Plant samples were harvested and washed in dilute detergent solution every 30 days, followed by several rinses in distilled water. Plant parts were dried in an oven at 70°C for 72 h, and the dry weights were recorded by electronic balance (the limit is 0.1 mg). Roots and shoots were digested, and the digestion was accomplished using an electric hot plate (Beijing, China) at 105°C for 30 min with 10 mL of concentrated HNO_3 (trace pure). Subsequently, the sample volume was adjusted to 20 mL with double deionized water and all sample extracts were analyzed using a flame atomic absorption spectroscopy (Spectra AA220, Varian, Mulgrave, Australia) (Boonyapookana et al. 2005).

For the processing of both plant and exudation samples, a series of standard samples were also prepared for the purpose of quality control, including: (1) analyzing 14 random samples, one blank sample and 1 standard sample each time; and (2) randomly selecting samples to ensure that the relative standard deviation were less than about 10%.

Statistical analysis. Statistical analyses were performed with the SPSS software package 17.0 (SPSS Inc., Chicago, USA). All data were subjected to the analysis of variance (ANOVA) and linear correlations among contents of metals, organic acids, pH, and redox potential using the Pearson's

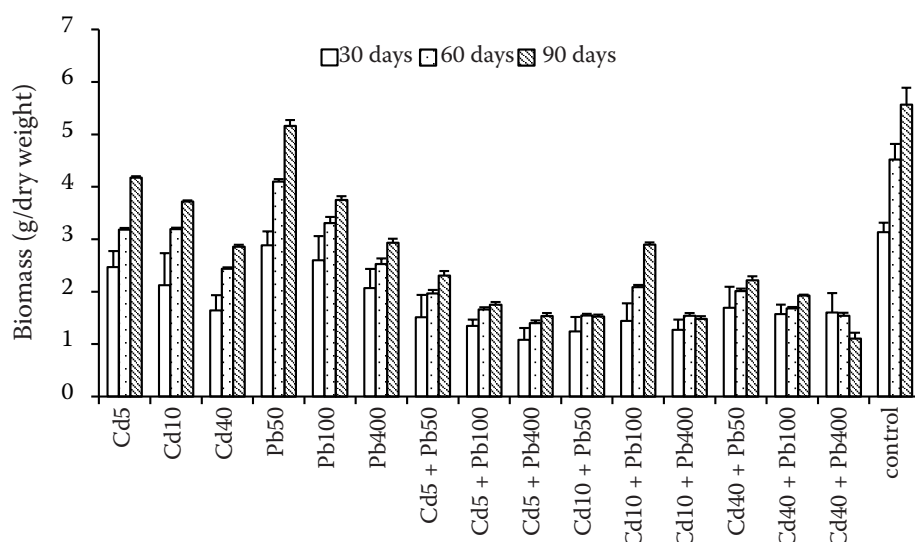


Figure 1. The biomass in the single and mixed treatments of Cd and Pb during 90 days (bars: standard deviation)

correlation coefficients. Differences at the $P < 0.05$ level were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Biomass and bioaccumulation of Cd and Pb.

In single treatments of Cd or Pb, biomass of sunflower decreased with the increase of Cd or Pb concentrations at the 30th and 90th day ($P < 0.05$) (Figure 1). The biomass in Pb treatments showed

higher than that in Cd treatments ($P < 0.05$). In comparison with the single treatments, the mixture of Cd and Pb significantly reduced the biomass of sunflower, and the highest biomass (2.8970 g) was found in Cd10 + Pb100 ($P < 0.05$).

In Cd treatments, Cd enrichment increased with concentrations and time (Figure 2). The highest Cd accumulation was 254.71 mg/kg in Cd40 at the 90th day ($P < 0.05$) (Figure 2a). In the mixed treatments, when Cd concentrations were 5 and 40 mg/kg, the addition of Pb decreased the Cd ac-

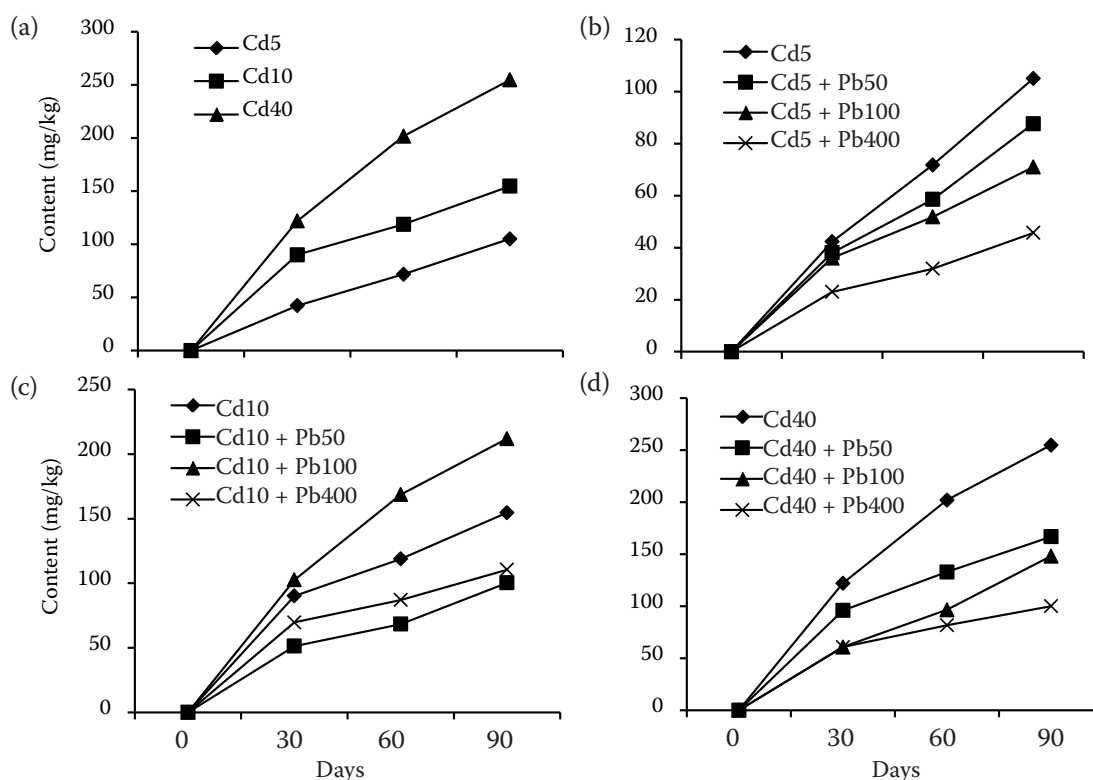


Figure 2. The contents of Cd accumulated by sunflower in the single and mixed treatments of Cd and Pb during 90 days (bars: standard deviation)

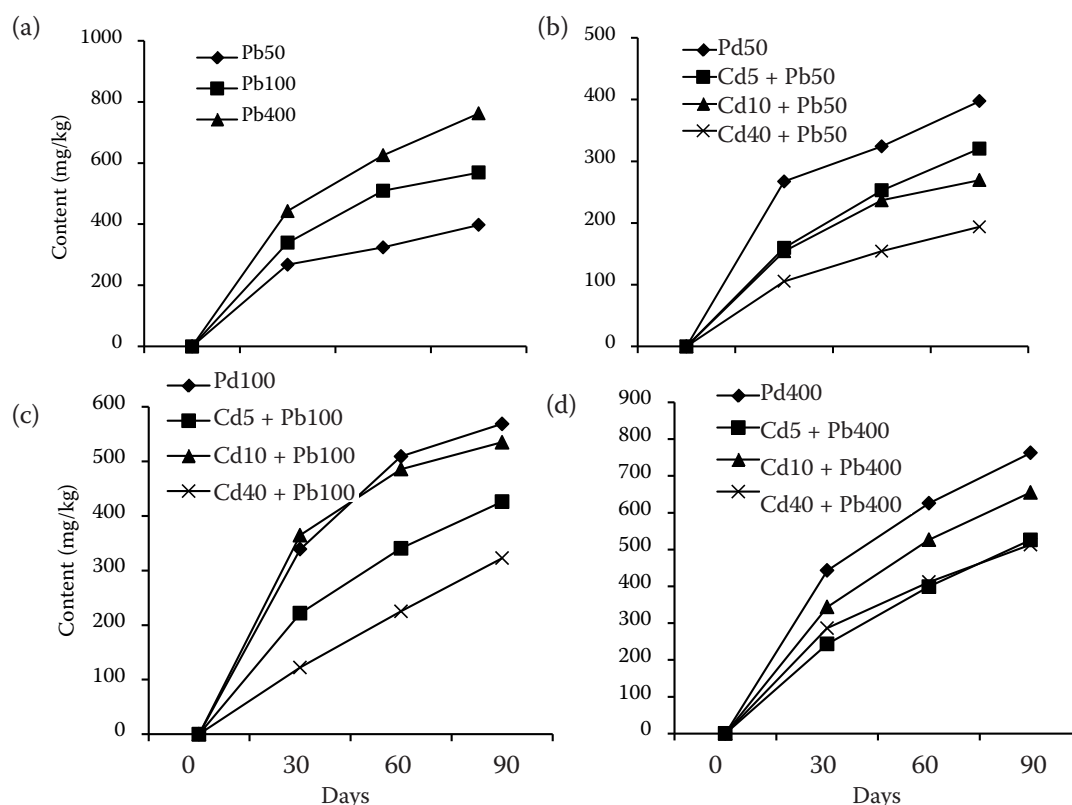


Figure 3. The contents of Pb accumulated by sunflower in the single and mixed treatments of Cd and Pb during 90 days (bars: standard deviation)

cumulation (Figures 2b,d). But in Pb100 + Cd10, 100 mg/kg Pb enhanced the Cd uptake (Figure 2c).

Pb contents in Pb treatments reached the highest at the end of the experiment (Figure 3a). When Pb was 100 mg/kg, the addition of Cd 40 mg/kg inhibited the Pb enrichment greater than treatments with 5 and 10 mg/kg Cd added (Figure 3c); in Pb400 plus Cd treatments, Pb accumulation showed no difference between Pb400 + Cd5 and Pb400 + Cd40 (Figure 3d).

Toxic metals could alter the growth of plants through disturbing the respiratory carbohydrate metabolism in plant cells by substituting irreversibly for another micronutrient in critical enzymes, in addition, interactions between enzymes and Cd or Pb might cause metals to exhibit deleterious effects on much of the biochemical machinery, including the formation of chlorophyll and the synthesis of aminoevulinic acid in plants (Paczkowska et al. 2007). Unavoidably, the changes of physiological processes in sunflowers owing to toxic metals would influence the phytoextraction (Boonyapookana et al. 2005). Moreover, the concentration of metals is a non-negligible factor to the bioaccumulation. Lu and He (2005) studied Cd enrichment in soil by *Ricinus communis* L. at 10 mg/kg to 400 mg/kg concentrations, results showed that the maximum accumulation ap-

peared at the concentration of 360 mg/kg but not 400 mg/kg. High concentrations could increase the content of heavy metals in tissue compartments of plants, but they weakened the accumulation ability conversely (Matés et al. 2010). In our study, the existence of one ion promoted the uptake of the other ion. This result was agreed with Lin et al. (2000), who reported that 5 mg/kg Cd improved the bioavailability of Pb in soil. Studies showed that the interaction of Cd and other metals, such as Zn and Pb, at equimolar concentration could overcome the toxicity of cadmium to plants (Chen et al. 2010).

Acidity and redox potential. During the 30th to 90th day, values of pH in Pb treatments were lower than those in Cd treatments (Figure 4a). In the mixed treatments, the acidity in Cd10 + Pb100 was lower (6.22–6.24) ($P < 0.05$), and pHs (6.72–6.75) in Cd10 + Pb50 were higher than the other treatments ($P < 0.05$). Redox potentials in single treatments showed opposite tendency with concentrations (Figure 4b). In the mixed treatments, the highest redox potential was 119.17 mv in Cd10 + Pb100 at the end of experiment ($P < 0.05$), and the values reduced when more Pb added at the same Cd concentrations, except for Cd10 plus Pb treatments.

Organic acids. The amount of glutaric acids decreased with concentrations mostly in treat-

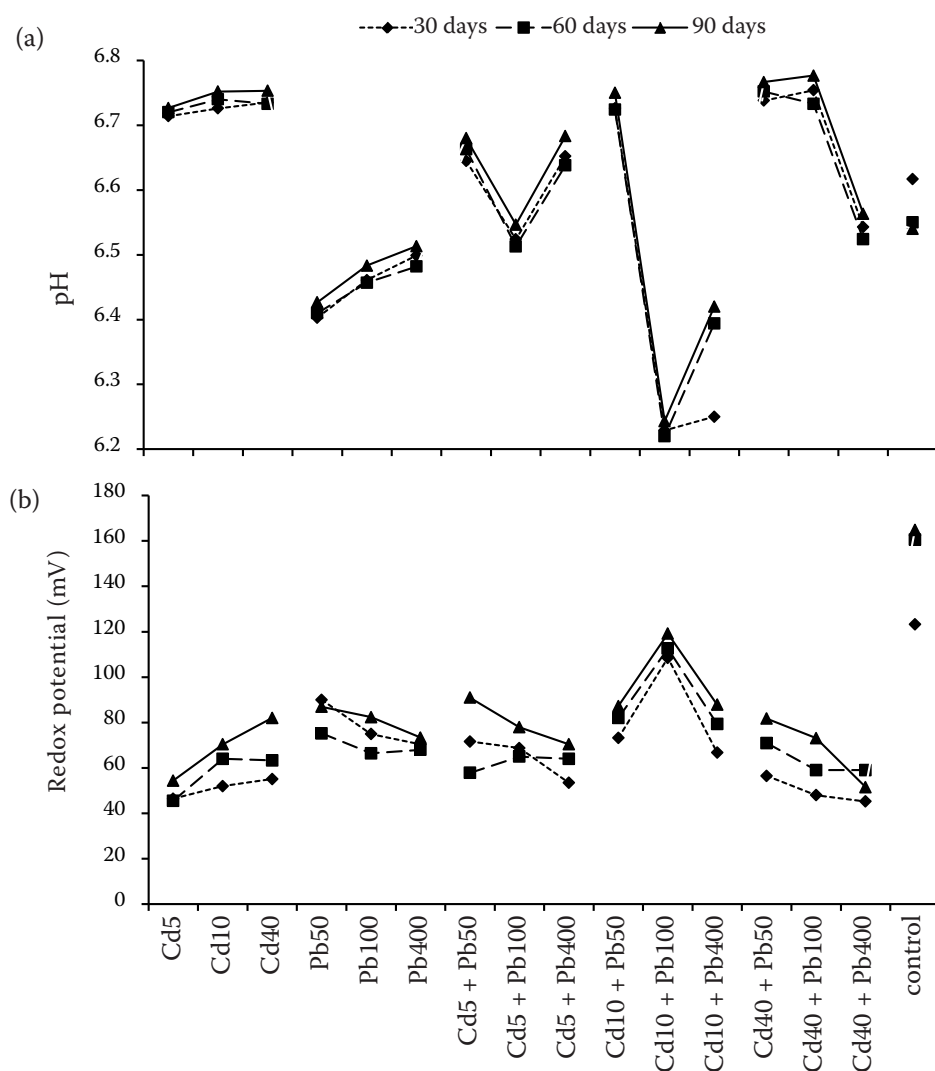


Figure 4. pH values (a) and redox potentials (b) in the single and mixed treatments of Cd and Pb during 90 days

ments (Figure 5a), and they were the highest in Cd5 and Pb50 treatments at the 90th day (440.0 mg/L, 364.00 mg/L, respectively) ($P < 0.05$). In the mixed treatments, glutaric acids in Cd5 plus Pb treatments declined with the Pb concentrations, and the contents in Cd5 + Pb50 and Cd10 + Pb100 (230.60 mg/L, 255.00 mg/L at the end of test) were more than those in other treatments ($P < 0.05$). The highest glycolic acid was 152.10 mg/L in Cd5 at the 90th day ($P < 0.05$) (Figure 5b). When Cd was 5 mg/L in the mixed treatments, glycolic acids contents decreased with Pb concentration; the amount of glycolic acids (217.00 mg/L) in Cd10 + Pb100 were more than the others ($P < 0.05$). Similar to glutaric acids, lactic acids showed decrement with concentrations in single treatments of Cd and Pb (Figure 5c). In the mixed treatments, the lactic acids increased with Pb addition when Cd concentrations were the same, and the highest appeared in Cd10 + Pb100 (232.10 mg/L) at the end of the experiment ($P < 0.05$).

Correlations analysis. In single treatments, Cd accumulated was negative to lactic acids ($r = -0.666$; $P < 0.01$), and Pb enrichment was positive to pH ($r = 0.397$, $P < 0.05$). Redox potential showed positive to glycolic acid and lactic acid ($r = 0.627$, $P < 0.01$; $r = 0.682$, $P < 0.01$), but negative to pH obviously (Table 1). In the mixed treatments, pH values displayed negative to Pb accumulation and redox potential significantly. Both Cd and Pb enrichment were positive to redox potential, glycolic and lactic acids, meanwhile, redox potential exhibited positive correlation with these three acids.

Roots of plants could secrete a diverse array organic acids to alter redox potential condition and acidity of media, as a result, toxic metals' availability to plants was affected directly (Zhang et al. 2009). The acidification of rhizosphere enhanced the Pb accumulation in this experiment. Marschner et al. (1989) considered that dicotyledons could acidify the rhizosphere via organic acid extrusion from the roots to increase metals accumulation. On the other

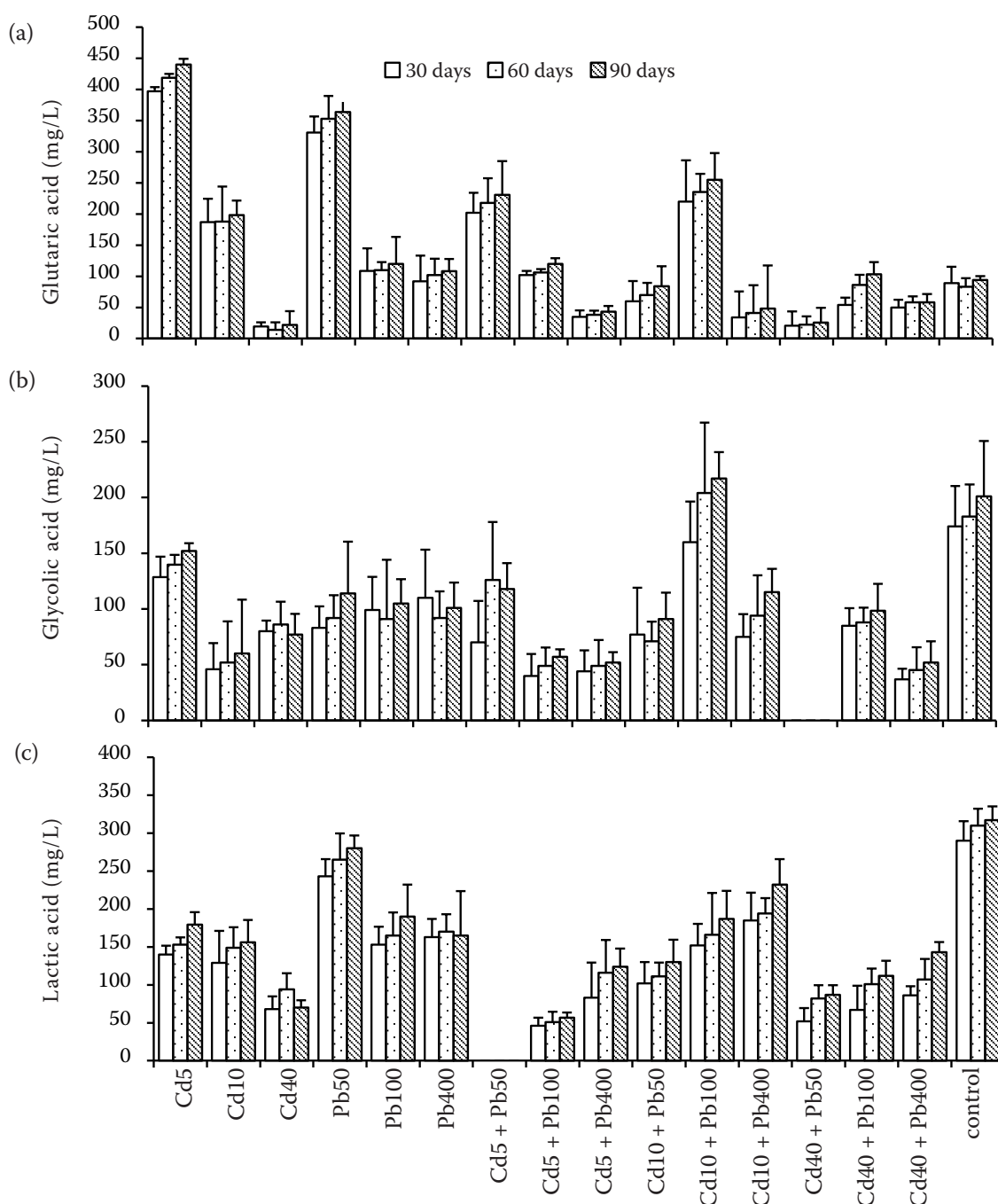


Figure 5. Contents of (a) glutaric acid; (b) glycolic acid and (c) lactic acid in the single and mixed treatments of Cd and Pb during 90 days (bars: standard deviation)

hand, pH values in single Cd treatments illustrated elevated trend in our study, which was consistent with the results of Zeng et al. (2011), who found that the pH increment made Cd more exchangeable and bioavailable in cultures. Furthermore, plants had been documented to catalyze redox reactions and alter the chemistry of metal ions. To avoid toxicity, redox potential in rhizosphere could transform or immobilize heavy metals (Levitt et al. 2011). Lytle et al. (1998) reported that *Eichornia crassipes* enriched nontoxic Cr^{3+} in root and shoots through

changing redox potential of rhizosphere in a solution with toxic Cr^{6+} . Andrade et al. (2004) deemed that the redox potential lowering would contribute to heavy metals found exclusively in insoluble forms. Exudation of organic acids by roots was one of the most important strategies developing by plants to tolerate the toxic metals, their influence on the phytoextraction depended on the reactions between metals and acids in rhizosphere (Cai et al. 2011).

Organic acids fluctuations under the stress of heavy metals appeared in our experiment, which

Table 1. Correlations among metals uptake, redox potential, organic acids and pH in the single and mixed treatments of Cd and Pb ($n = 144$)

		Redox potential	Glutaric acid	Glycolic acid	Lactic acid	pH
Single Cd, Pb treatments	Cd-content	-0.208	-0.193	-0.356	-0.666**	0.157
	Pb-content	-0.260	-0.236	-0.020	0.247	0.397*
	redox potential	1	0.188	0.627**	0.682**	-0.925**
Mixed treatments of Cd and Pb	Cd-content	0.645**	0.299	0.449*	0.433*	-0.160
	Pb-content	0.425*	0.160	0.462*	0.680**	-0.471**
	redox potential	1	0.610**	0.717**	0.423*	-0.344**

** $P < 0.01$; * $P < 0.05$

were addressed in many articles. In the study of Zhang et al. (2009), the secretion of glycolic, citric, and succinic acids in tomato rhizosphere was disturbed by extraneous gadolinium; Greger and Landberg (2008) found that Cd concentration elevation in soil decreased levels of lactic and citric acids in wheat root exudates. While, Chiang et al. (2006) pointed that acetic, lactic, and glycolic acids in the rhizosphere of tobacco and sunflower increased with increasing amendment of Cd (1, 5, 10 mg/L); and Cd (less than 5.0 mg/L) could enhanced the malate exudation of sorghum, and citrate exudation of maize (Pinto et al. 2008), which meant that the composition of the organic acids from roots and the toxicity symptoms were specific to plants and heavy metals (Smith et al. 2011). Heavy metals could lead to fluctuations of major nutrient cations in the root cells and exudates, either by co-release with organic acid anions or by displacements in the cell walls and on the plasma-membrane surfaces (Kochian et al. 2005). Besides, alteration of some element pool in the cells induced by metals could stimulate callose formation in roots (Hirano et al. 2012), and callose is a plant polysaccharide that is formed in response to various stress factors such as heavy metals (Krzesłowska 2011). The mechanism on changes of root exudates attributed to heavy metals interference is complicated and more studies are needed.

In our study, sunflower was applied to enrich cadmium and lead in sand culture. The accumulation ability of Cd and Pb was affected by species and concentrations of metals. Moreover, the presence of Cd and Pb influenced the lactic, glutaric, and glycolic acids in root exudation variously. Rhizospheric conditions, including acidity and redox potential, could be disturbed by changes of organic acids, which made the bioavailability and phytoextraction of toxic metals impacted.

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